A Short-Term *In Vivo* Screening System for Endocrine Disruptors Utilizing Mosquitofishes (*Gambusia affinis* and *G. holbrook*)

Project Scope

Female mosquitofish can be useful bioindiators in helping to identify compounds that effect endocrine systems at environmentally-relevent concentrations. Because mosquitofish are small, easily bred, and have short life and reproductive cycles, they can be used to develop short-term in vivo and in vitro tests for hormonal activity that are fast, reliable, and inexpensive.

Several studies have demonstrated the usefulness of characterizing the responses of female mosquitofish to substances with androgenic activity and have provided basic dose-response data on the masculinizing effects of various androgens on female mosquitofish.

The purpose of this research was to establish and document the use of mosquitofish (*Gambusia affinis* and *G. holbrooki*) as highly sensitive bioindicator organisms for use in screening substances with suspected endocrine activity, either singly or in complex mixtures. Expression of the (normally female-limited) vitellogenin gene in males was used to test chemicals for estrogenic activity. A morphological trait (modified anal fin called a gonopodium) was used to test for androgenic activity. The gonopodium is not found in normal females, but is readily induced to develop in females exposed to chemicals with androgenic activity. In addition to their utility as laboratory test organisms, these fish were shown to be suitable as sentinel species for the detection of endocrine disruptors in a variety of fresh- and warm salt-water environments.

To address these objectives the project sought to: 1) develop an assay for vitellogenin (VTG) that could be used to detect this egg yolk protein in the serum of male mosquitofish; 2) develop a quantitative index of the effects of androgenic chemicals on the anal fin morphology of females; 3) perform dose-response experiments on the effects of estrogenic organochlorine insecticides (singly, and in mixtures) on VTG expression in male mosquitofish; 4) perform dose-response experiments on the effects of androgenic chemicals on the anal fin morphology of females; 5) investigate the effects of prior hormone exposures on reproductive function of female mosquitofish; and 6) perform laboratory and field tests of selected potential endocrine disruptors.

Grant Title and Principal Investigator

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Key Findings and Implications

- A solid phase (Western blot) immunoassay for vitellogenin (VTG) was developed, which was used to demonstrate that the effect of 17α-ethynylestradiol on VTG synthesis in male *G. affinis* is both concentration- and time-dependent.
- When coupled with densitometric scanning, the solid phase assay appeared to be specific, quantitative, and sensitive enough to detect VTG down to 10 ug/g in *G. affinis* blood.
- A quantitative morphological study of gonopodium development in normal males and in females treated with 11-ketotestosterone showed that length and width ratios of anal fins are useful in that they can be determined with high precision and are not restricted to a limited size range.
- Phytosteriods in paper mill effluent tend to accumulate in the sediment, where microbes convert them first into progesterone and then into androstenedione and other bioactive steroids. Also, it was found that unpolluted water bodies with much lower organic matter still contain progesterone, but at substantially lower levels.
- No histological changes in the testes or liver were observed in male mosquitofish near a wastewater treatment plant that would indicate exposure to estrogens and no detectable levels of VTG were found in their blood.

Project Period: October 1997 to September 2000

Relevance to ORD's Multi-Year Research Plan

This project contributes to ORD's Multi-Year Plan long-term goal of determining the extent of the impact of endocrine disruptors on humans, wildlife, and the environment by using mosquitofish as highly sensitive bioindicator organisms for screening substances with suspected endocrine activity. The goal is to be able to screen potential endocrine disrupting substances either singly or in complex mixtures. This research contributes to addressing this goal by helping to develop a short-term screening system that reflects both exposure to, and effects of endocrine disruptors; increasing knowledge on latency and effects of prenatal exposure on reproductive characteristics of adults; improving information on the effects of low-dose exposures reflecting realistic environmental levels; and providing data on the effects of mixtures of chemicals in an in vitro test system.

Project Results and Implications

Vitellogenin as a Biomarker for Estrogens. Vitellogenin (VTG) was first identified and purified and then an anti-VTG immume serum was produced and characterized using Western blot. Analysis revealed that antibodies in the immume serum bound specifically to the protein previously identified as VTG, but did not bind to any other protein in male or female blood. Also, when blood samples were probed with pre-immune serum, no binding was observed.

To determine if this immunoblotting would be useful for monitoring the effect of estrogenic compounds on vitellogenesis, Western blotting was coupled with densitometric scanning to assess the dose- and time-dependency of 17a-ethynylestradiol (EE_2)-induced vitellogenesis in male mosquitofish. VTG immunoreactivity was detected in blood samples from fish treated with EE_2 doses as low as 10 ug/g of food. The assay has been used to demonstrate that the effect of EE_2 on vitellogenin synthesis in male *G. affinis* is both concentration- and time-dependent.

Assay for Gonopodium Development in Females. A quantitative morphological study and dose-response experiment of gonopodium development in normal males and in females treated with doses of 11-ketotestosterone was completed using computer image analysis techniques. The length and number of anal fin rays were used to quantify the degree of gonopodial development, both in normal males and androgen-treated females, and results of doses ranging from 20 to 100 ug of hormone/g of food were examined. The morphological results showed that the length and width ratios of anal fins are useful in that they can be determined with high precision and are not restricted to a limited size range. Dose-response results show that at the higher doses (60, 80, and 100 ug/g), the androgen-treated females all developed gonopodium-like characters in the anal fins, whereas at the lower doses (20 and 40 ug/g), one of the four fish at each concentration did not respond at all. The rapidity of response and extent of elongations differed substantially among the doses given, but the response was largely all-or-none (i.e., the fish either did or did not exhibit changes in the anal fins), especially at the lower doses.

Identifying the Androgenic Components of Paper Mill Effluent. A toxicity identification and evaluation approach was used to identify the androgenic components of paper mill effluent in the water column and sediment downstream from a mill in Florida. The river was chosen because of the occurrence of masculinized mosquitofish. For the water samples, the approach consisted of fractionating water extracts, performing in vitro assays to locate bioactive fractions, and identifying compounds using mass spectrometry. For the sediment, the approach involved solid phase extraction and high pressure liquid chromatography purification, androgen receptor transcription assays, and liquid chromatography mass spectroscopy.

All of the female fish collected (*G. holbrooki* mosquitofish, sailfin molly, and killfish) from the river containing effluent from a nearby paper mill were masculinized. Androstenedione was determined to be a major component of the river water, at a concentration of 0.14 nM. Androstenedione was found in the sediment at a concentration of 2.4 nM or 17 times greater than measured in the water column, indicating that androgens that are measured at low concentrations in the water have the potential to contain much high sediment concentrations. In addition, a biosynthetic precursor of androstenedione, progesterone, was measured in the sediment at 155 nM, which is a concentration 65 times greater than androstenedione in the sediment and 1000 times greater than that found in the water for progesterone. It is therefore hypothesized that the phytosteriods in the paper mill effluent tend to accumulate in the

sediment, where microbes convert them first into progesterone and then into androstenedione and other bioactive steroids. Also, it was found that unpolluted water bodies not downstream from mill effluent and with much lower organic matter still contain progesterone, but at substantially lower levels.

Reproductive Characteristics of Male Mosquitofish. Sexually mature male western mosquitofish (*G. affinis*) were collected downstream of a large wastewater treatment plant near Birmingham, Alabama to determine effects of exposure to estrogens. Although testes and liver weights were higher in the effluent-exposed population, no histological changes in the testes or liver were observed in the study that would indicate exposure to estrogens and no detectable levels of VTG were found in the blood of the male mosquitofish.

In addition to the work on the project already described, the following is planned:

- Dose-Response Assays With Androgenic Chemicals. These studies to characterize the relative sensitivity of the female anal fin rays to a variety of steroidal and nonsteroidal chemicals will continue.
- Reproductive Effects of Masculinization in Females. Female mosquitofish of reproductive age are being masculinized by exposure to methyltestosterone. Then, the effects of the androgen exposure on their reproductive ability will be measured to examine the effects on ovarian weight and morphology, histology, female fecundity (brood frequency and size), and survivorship of the young after birth.
- Effects of Masculinizing Chemicals on Embryos. Pregnant females will be treated with masculinizing chemicals, exposing the embryos in utero. The young will be raised to maturity so that the effects of the chemical treatments on sex ratio and fertility can be assessed.

Investigators

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For More Information

http://www.uab.edu/uabbio/angus.htm

NCER Project Abstract and Reports:

http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/173/report/F